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# Reactivity of the Alkaline Phosphatases of Bovine Milk and Intestinal Mucosa with the Substrates Phenyl Phosphate and o-Carboxyphenyl Phosphate

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Bovine intestinal mucosa alkaline phosphatase reacts equally with the substrates phenyl phosphate and *o*-carboxyphenyl phosphate. Milk alkaline phosphatase, on the other hand, at a concentration of the substrates of about $8 \times 10^{-5}$ moles/l., is $^1/_3$ to $^1/_4$ as reactive with the *o*-carboxyphenyl phosphate as with phenyl phosphate. Analyses of these reactions with a range of substrate concentrations showed that the difference in reactivity is due to a difference in enzyme–substrate dissociation constants, $K_m$ . The p$K_m$ values for the mucosa phosphatase with both substrates at pH 9.7 is 3.7; with the milk phosphatase and phenyl phosphate the p$K_m$ value is 4.6, whereas with *o*-carboxyphenyl phosphate the value is 2.8. The $K_m$ values were also determined for a range of pH values. The difference noted above persisted throughout. $K_m$ values obtained with the milk phosphatase in a lipide-containing complex were identical with values obtained with the phosphatase after dissociating with *n*-butyl alcohol.

## INTRODUCTION

Quantities of bovine milk phosphatase and intestinal mucosa phosphatase that gave equal hydrolysis of the substrate phenyl phosphate behaved differently with the substrate *o*-carboxyphenyl phosphate. The mucosa phosphatase was equally reactive with both substrates, but the milk phosphatase was only about $^1/_4$ as reactive with *o*-carboxyphenyl phosphate. The present report describes kinetic studies with varying substrate concentrations and pH values to determine the factors responsible for the difference in reactivity. At the same time, a comparison has been made of the behavior of the milk phosphatase as it exists in a lipide-containing complex which may represent mammary tissue microsomes (1–3) with the milk phosphatase obtained by dissociating the complex with *n*-butyl alcohol (4).

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## MATERIALS AND METHODS

### Intestinal Mucosa Alkaline Phosphatase

Preparation of this material from the intestinal mucosa of the calf has been described (5). It has been used in a number of previous studies (6, 7).

### Milk Alkaline Phosphatase

Three preparations of this enzyme, differing in physical complexity, have been used. (*a*) The phosphatase in its native lipoprotein complex was prepared essentially by a method that has been described (3). Casein was removed from skim milk with rennin. The whey was precipitated with 2.3 *M* ammonium sulfate. The wet salt cakes were stored at 7°. Ten grams of the cake was dissolved in 100 ml. of 0.1 *M* ethanolamine–HCl buffer, pH 9.4, and centrifuged at $2000 \times g$ for 10 min. to remove gross particles, and the supernatant solution was centrifuged at $127{,}000 \times g$ for 30 min. The small amount of sediment obtained was dispersed in 10 ml. of 0.1 *M* tris(hydroxymethyl)-aminomethane–HCl buffer, pH 8.1, and clarified by centrifuging at $2000 \times g$ for 10 min. This solution, used for the studies described, contained

40% of the phosphatase in the starting solution. (*b*) This phosphatase was prepared by a method employing trypsin (6) which partially decomposed the lipoprotein complex. It was prepared as described (6), dried from the frozen state and stored at 7°. (*c*) This phosphatase was prepared by dissociating the lipoprotein complex with *n*-butyl alcohol and further fractionated with acetone, followed by Filter-Cel adsorption of impurities (4). Dry products were obtained by precipitation with acetone.

## Phosphatase Assays

(*a*) *Substrate: Phenyl Phosphate.* The reaction was performed in 0.067 *M* ethanolamine buffer at 37°, and the phenol released was determined colorimetrically. Details of the procedure have been described (6). The blue color obtained was read in a Beckman model B spectrophotometer in 18 mm. O.D. tubes at a wavelength of 650 mμ.

(*b*) *Substrate: o-Carboxyphenyl Phosphate.* The properties of this substrate and its usefulness for the assay of alkaline phosphatase has been described by Brandenberger and Hanson (8) and by Hofstee (9). Several procedures were used in the present study. The most convenient procedure with this substrate is to carry out the reaction in the Beckman model DU spectrophotometer cell maintained at 38° with thermospacers, and to take absorbance readings at 310 mμ at several time intervals as the reaction proceeds. This procedure can be varied if the phosphatase solutions are turbid, which is the case in the early stages of preparing the phosphatase. The reaction is performed at 38°, stopped by the addition of trichloroacetic acid to give a concentration of 10% (*v/v*), clarified by centrifugation, and the increase in optical absorbance read at 310 mμ.

Since the molar absorbance of *o*-carboxyphenol at 310 mμ (2600 in the presence of magnesium ion) was considerably less than that developed with phenol (approximately 7500), in general for the best results it was necessary to use five times the amount of enzyme with the *o*-carboxyphenyl phosphate substrate. For the best comparison on an equal phosphatase basis, the liberated *o*-carboxyphenol was determined colorimetrically with the phenol reagent (6); the resulting color was one-third of that obtained with phenol. In a few experiments the liberated inorganic phosphate was determined. This was done in a comparison of the substrates phenyl and *o*-carboxyphenyl phosphates, together with serine phosphate.

Magnesium chloride was added to all the assay mixtures to obtain the full activity of the phosphatases; the final concentration of magnesium ion was 0.0025 *M*. The magnesium ion increases the absorption of *o*-carboxyphenol at 310 mμ at pH 10 about 50%,[2] and must be considered in equating concentration of *o*-carboxyphenol liberated with the absorption at 310 mμ. The effect was negligible below pH 10.

*Determinations of pH.* The pH values were all determined at 25° with the glass electrode, regularly standardized against known buffers.

## Calculations of Results

Spectrophotometer readings are converted to equivalent moles of split product phenol or *o*-carboxyphenol and expressed as moles per 5 min. in a reaction volume of 6 ml. Measurements are made at 5- and 10-min. intervals, or at more frequent shorter time intervals in the direct spectrophotometric method, to determine whether the reaction is proportional to time, presumably indicating independence of substrate concentration. Where the rates changed with time, they were found to be first order and obeyed the equation $kE = (2.3/t)(\log_{10}C_0/C_t)$ (10) where $k$ is the rate constant, $E$ a constant concentration of enzyme, $C_0$ is the concentration of substrate initially, $C_t$ is the concentration of substrate at time $t$. $kE/2.3$ is conveniently evaluated from the slope of the plot of log $C_0/C_t$ versus $t$. The rate $(-dC/dt)$ at zero time is calculated with the rate constant $k$ in the equation $-dC/dt = kEC$ where $C$ is the concentration of substrate. When low concentrations of *o*-carboxyphenyl phosphate were used as substrate, the reaction followed the first-order equation. By taking Beckman readings at short time intervals (15 sec.), initial reaction rates were in agreement with those computed from the first-order equation.

## RESULTS

The results in Table I show that, at pH 9.7, the intestinal phosphatase hydrolyzes both substrates at similar rates, whereas the milk phosphatase (*b*) is less active with *o*-carboxyphenyl phosphate than with phenyl phosphate. Similar results were obtained with the soluble milk phosphatase (*c*) in experiments in which both phenol and *o*-carboxyphenol were determined colorimetrically. In another series of experiments, which also included serine phosphate as a substrate,[3] the extent of hydrolysis was measured by the inorganic phosphate released.

[2] Zittle, C. A., Bingham, E. W., and Pepper, L., unpublished studies.

[3] Zittle, C. A., and Bingham, E. W., unpublished studies.

Again the hydrolysis of *o*-carboxyphenyl phosphate by the milk enzyme was only about ¼ that with the phenyl phosphate. On the other hand, the ratio of mucosa to milk phosphatases (4 to 100 in this experiment) giving equal hydrolysis of phenyl phosphate also gave equal hydrolysis of serine phosphate.

In view of the above results, similar experiments were performed at several substrate concentrations. These data permitted the calculation of $K_m$, the dissociation constant for the substrate–enzyme complex, and $V$, the limiting rate at high substrate concentrations, from the formulation (10) $1/v = K_m/VC_0 + 1/V$. $v$ is the initial rate of reaction and $C_0$ is the initial concentration of substrate. Plots of $1/v$ versus $1/C_0$ are prepared for which the slope of the straight line is $K_m/V$ and the intercept is $1/V$.

The separate evaluation of initial rates of reaction for the calculation of $K_m$ and $V$ by the above procedure can be avoided by the use of the equation developed by Walker and Schmidt (11) by integration of the above equation:

$$1/t[2.3 \log S_0/S_t] = (-1/K_m)(S_0 - S_t)/t + V/K_m$$

$S_t$ is substrate concentration at time $t$. By a plot of $1/t[2.3 \log S_0/S_t]$ versus $(S_0 - S_t)/t$, a straight line is obtained whose slope is $-1/K_m$ and the intercept on the $(S_0 - S_t/t$ axis is $V$. The use of this equation is discussed by Dixon and Webb (12).

Figure 1, which represents typical results, shows the relationship between the rate of hydrolysis and substrate concentration for the milk phosphatase (*c*) with phenyl and *o*-carboxyphenyl phosphates. It is evident that the association between the *o*-carboxyphenol substrate and the enzyme is much weaker than with the phenol substrate. The hydrolysis of *o*-carboxyphenyl phosphate was linear with time, and when the data are converted to moles per liter and plotted as described earlier ($1/v$ versus $1/C_0$) a value of $K_m$ of 0.00116 is obtained. This value, converted to p$K_m$ of 2.94, is shown in Fig. 2 with results at other pH values. The hydrolysis of phenyl phosphate with two concentrations of phosphatase (0.02 and 0.01 mg.) showed the influence of the depletion of substrate. The results fitted the first-order equation for calculating $v_0$ which could be used in the above type of plot, or the results were plotted directly by the Walker-Schmidt procedure. The $K_m$ value obtained was

TABLE I

REACTIVITY OF BOVINE INTESTINAL MUCOSA ALKALINE PHOSPHATASE AND MILK ALKALINE PHOSPHATASE WITH THE SUBSTRATES PHENYL PHOSPHATE AND *o*-CARBOXYPHENYL PHOSPHATE

Experimental conditions: concentration of substrates 450–500 × $10^{-8}$ moles in volume of 6.0 ml.; pH 9.7 maintained by 0.067 *M* ethanolamine–HCl buffer; temperature 37°.

| Phosphatase preparation | Amount of phosphatase used | Substrate | Hydrolysis |
|---|---|---|---|
| | *μg.* | | *moles ($10^{-8}$)/ 5 min.* |
| Mucosa | 20 | Phenylphosphate | 59.2 |
| Milk (*b*) | 1000 | Phenylphosphate | 60.8 |
| Mucosa | 20 | *o*-Carboxyphenyl-phosphate | 72.6 |
| Milk (*b*) | 1000 | *o*-Carboxyphenyl-phosphate | 17.0 |

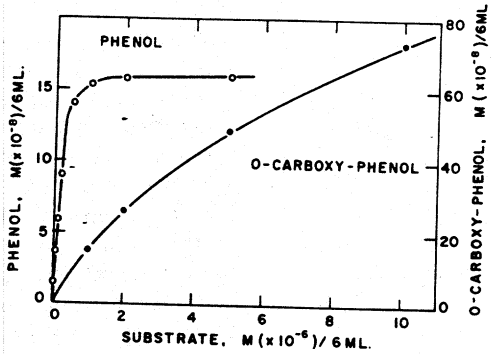


FIG. 1. Hydrolysis of phenyl phosphate and *o*-carboxyphenyl phosphate by milk phosphatase (*c*) at pH 9.7 with several concentrations of the substrates at 37°. Hydrolysis is expressed as moles of phenol or *o*-carboxyphenol released in 5 min. in volume of 6 ml. Phenol released was measured colorimetrically, *o*-carboxyphenol released was measured from the change in absorption at 310 mμ as the reaction proceeded in the absorption cell. Milk phosphatase used: 0.02 mg. with the phenyl-phosphate, 0.10 mg. with the *o*-carboxyphenyl phosphate.

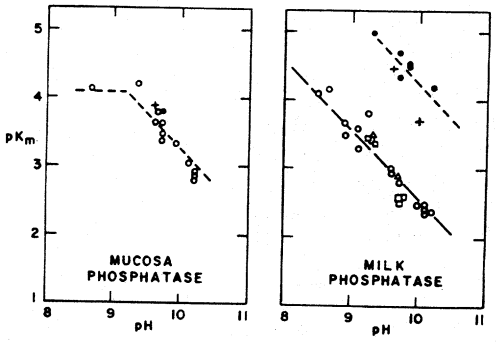


FIG. 2. Enzyme–substrate dissociation constants ($pK_m = \log 1/K_m$) for the mucosa and milk phosphatases acting on the substrates phenyl phosphate and *o*-carboxyphenyl phosphate at several pH values. *Mucosa phosphatase:* with *o*-carboxyphenyl phosphate ○. For comparison two values obtained with phenyl phosphate are shown: ●, present work; +---, Zittle and DellaMonica (6). *Milk phosphatases:* preparation (*a*) with *o*-carboxyphenyl phosphate △; preparation (*b*) with *o*-carboxyphenyl phosphate □, (*b*) with phenyl phosphate +---, Zittle and DellaMonica (6); preparation (*c*) with *o*-carboxyphenyl phosphate ○, (*c*) with phenyl phosphate ●.

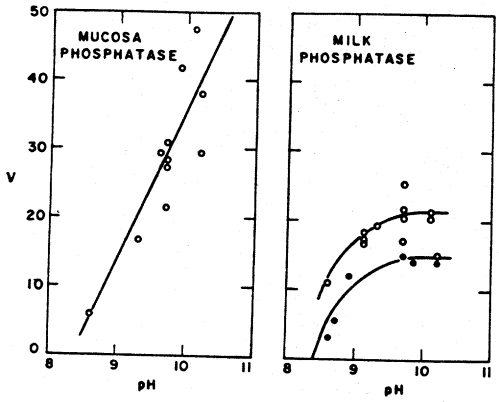


FIG. 3. The maximum velocity hydrolysis ($V$) of the substrates phenyl phosphate and *o*-carboxyphenyl phosphate by the mucosa and milk phosphatases at various pH values at 37°. The $V$ values are expressed as moles/l./5 min./unit weight of phosphatase. For the *mucosa phosphatase* the weight of enzyme was 1.3 mg. (per liter); for the *milk phosphatase* with the substrate *o*-carboxyphenyl phosphate (○), the unit weight was 330 mg.; with the phenyl phosphate (●), the enzyme preparation used was considerably more active and the unit weight was 3.3 mg.

0.0000174, or a $pK_m$ of 4.76. Thus the affinity of the milk phosphatase for phenyl phosphate is about 100-fold greater than for *o*-carboxyphenyl phosphate.

Since the difference in affinity of the milk phosphatase for the two substrates might be due to differences in ionization at the substrate binding sites, the influence of pH on $K_m$ and $V$ (12, 14) was also studied to obtain additional information concerning the two enzymes. The variation of $pK_m$ with pH and $V$ with pH are shown in Figs. 2 and 3, respectively.

## DISCUSSION

The milk phosphatase hydrolyzes *o*-carboxyphenyl phosphate ⅓ to ¼ as fast as phenyl phosphate when the concentration of each substrate is about $8 \times 10^{-5}$ moles/l. This is found to be due to a difference in the affinity of the milk phosphatase for each substrate. The milk phosphatase is much more firmly bound to the phenyl phosphate than to the *o*-carboxyphenyl phosphate as shown by the results in Figs. 1 and 2. At about fourfold higher concentration of the substrates ($32 \times 10^{-5}$ moles/l.) the milk phosphatase reacts equally with both substrates. The mucosa phosphatase reacts equally with both phenyl phosphate and *o*-carboxyphenyl phosphate at all concentrations, and, accordingly, $K_m$ values are the same (Fig. 2). Since the phenol and *o*-carboxyphenol substrates differ principally in the negative charge on the latter, the observed poor affinity of the milk phosphatase with the *o*-carboxyphenyl phosphatase might be due to a negative charge on the enzyme in the neighborhood of the substrate binding site, leading to repulsive effects. The phosphatase reaction, however, was not altered by performing it in the presence of 0.1 $M$ NaCl. It is of interest that certain more complex anions are more inhibitory to the mucosa phosphatase than to the milk phosphatase, whereas with certain cations the reverse is true (7).

The effect of changes in pH on the enzyme-dissociation constant showed some differences (Fig. 2) between the two enzymes. Differences also appear in the $V$ versus pH curves (Fig. 3). The $pK_m$ versus pH curve

for both phosphatases with both substrates have a slope of minus one down to pH 9.2, indicating that a change of one unit in charge is involved in formation of the enzyme–substrate bond (14). With the mucosa phosphatase and the substrate *o*-carboxyphenyl phosphate the $pK_m$–pH slope decreases to zero at pH 9.2, similar to its behavior with phenyl phosphate reported by Morton (13). This property of the $pK_m$–pH curve indicates that the group participating in the binding of substrate has a $pK$ of 9.2, thus confirming the earlier finding of Morton (13, 14).

Morton (13) reported the $K_m$ values for phenyl phosphate and purified milk phosphatase at pH's 9.6 and 10.0. These values were in close agreement with those obtained by Folley and Kay (15) with a crude extract of guinea-pig mammary gland. The values of Morton (13) and Folley and Kay (15) show a $-1$ unit slope for the plot $pK_m$ versus pH, as found in the present study (Fig. 2). However, on the basis of a single determination by Folley and Kay (15) at pH 8.9, Morton (13) suggested that it was probable that a discontinuity occurred with milk phosphatase at about pH 9.2. The present results with milk phosphatase and the substrate *o*-carboxyphenyl phosphate show no such break in the $pK_m$–pH curve even down to pH 8.5. The present experiments with phenyl phosphate in this low pH range were not considered satisfactory because of the low enzyme activity and particularly the very low concentrations of substrate ($<2 \times 10^{-5}$ moles/l.) required for data for $K_m$ calculations.

Bamann and Riehl (16) observed that the $1/K_m$ values for bovine liver alkaline phosphatase acting on phenyl phosphate and several other substrates increased with pH, whereas values for several acid phosphatases decreased. They concluded that the substrate was most strongly bound to the enzyme when the phosphate portion was doubly charged. The present studies as well as those of others (13, 15, 17) indicate that this is not generally true. The present results indicate that the change in charge resulting from enzyme–substrate interaction leads to a decrease in enzyme–substrate affinity at higher pH values, whereas increase in pH increases the substrate to products breakdown (high $V$).

The data in Fig. 2 for the milk phosphatase preparations show that there was no difference in the affinity of the *o*-carboxyphenyl phosphate for the milk phosphatases differing in complexity; $pK_m$ values for the lipide complex (*a*), which may represent microsomes (1, 2), the butyl alcohol-dissociated preparation (*c*), and the partially dissociated preparation (*b*), were all the same.

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